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A REPORT ON THE PRODUCTION OF 2,3-BUTYLENE GLYCOL FROM WHEAT AND WHEAT STARCH

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University of Alberta

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A. Lorne Shewfelt
Department of Field Crops

A THESIS
submitted to the University of Alberta
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requirements for the degree of
MASTER OF SCIENCE

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INTRODUCTION

In cooperation with the National Research Council Laboratory, Ottawa, Ontario, and the Northern Regional Research Laboratory, Peoria, Illinois, the Department of Field Crops and the Department of Soils, University of Alberta in May, 1942, began a research project designed to study the yields of 2,3-butylene glycol from wheat. In all cases the conversion was accomplished through bacterial fermentation.

The fermentation work was divided into two distinct channels: (1) the production of butylene glycol from glucose, hydrolysed wheat, and hydrolysed wheat starch by butylene glycol producing organisms, chiefly Aerobacter aerogenes; (2) the production of butylene glycol from wheat and wheat starch by starch-hydrolysing bacteria.

The fermentation mixtures were prepared and the fermentations carried out in two separate laboratories at the university. When fermentation was complete the mixtures were transferred to a third laboratory for analysis. Quantitative estimation of butylene glycol, acetylmethylcarbinol

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The fermentation mixtures were prepared and the fermentations carried out in two separate laboratories at the university. When fermentation was complete the mixtures were transferred to a third laboratory for analysis. Quantitative estimation of butylene glycol, acetylmethylcarbinol

and residual sugar were conducted on the supernatant liquid of each fermentation mixture. Centrifuging of the mixtures was sometimes necessary in order to obtain a clear supernatant liquid, but generally the residues settled sufficiently so that suitable samples could be readily drawn off with a pipette.

This report does not include details of all the work conducted on the above project at this institution, but is presented as a summary of some of the more important phases in relation to the general progress which has been made. The author wishes to point out also that the project is being continued and, should the reader wish further information, he should look for subsequent reports by members of departments collaborating in this research.

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PART I

METHODS OF ANALYSIS

The methods of analysis used were essentially those recommended by the Northern Regional Research Laboratory at Peoria, in their original instructions (3). For the most part the fermentation liquors were analysed soon after the organism ceased working. Storing the samples for three or four days in a refrigerator had little effect on the results except for a slight increase in acetylmethylcarbinol at the expense of the glycol.

Estimation of Reducing Sugars

Sugar analyses were carried out according to a modification of the Shaffer-Hartmann method (4). Fehling's solution was used at first as an oxidative reagent but was later abandoned in favor of Benedict's reagent. The size of sample and amount of $KI-KIO_3$ solution were varied according to the amount of sugar expected to be present. The amount of copper reduced was determined iodometrically and converted to percentage glucose.

Part I

Methods of Analysis.

The methods of analysis used were essentially those recommended by the Microchemical Research Laboratory at Peoria, in their original instructions (1). The most part the fermentation liquors were analysed immediately after the organism ceased working. Storing the samples for three or four days in a refrigerator had little effect on the results except for a slight increase in acetylmethylcarbinol at the expense of the glycol.

Estimation of Residual Sugars

Residual sugars were determined by the Bertrand method (2). Initially a solution was made up to contain 1% sugar. Later this was abandoned in favor of Brown's method (3). The clear or sample and aliquots of 1%-10% solutions were boiled with Fehling's solution for two minutes. The cuprous oxide precipitate was determined iodometrically and converted to dextrose equivalents. The amount of sugar consumed was determined by the difference between the original and residual sugars.

Estimation of Acetylmethylcarbinol

Acetylmethylcarbinol ($CH_3.CHOH.CO.CH_3$), presumably a precursor of 2,3-butylene glycol ranged from 0.0 to 0.50% in the fermentation liquors. For the most part its presence constituted little more than a correction factor and with many liquors such corrections were negligible.

A qualitative test for the carbinol was performed on each liquor according to the method of Dorner and Hellinger (1). This eliminated the need for making a complete determination on every liquor since many contained mere traces of this material. Complete quantitative analyses for carbinol were necessary only on a small proportion of runs.

Extraction of Butylene Glycol from the Sample

For a considerable time ether extractions similar to those described in the original instructions were conducted on all liquors. Though no serious difficulty was encountered in this stage, it was time consuming, and occasional foaming resulted in part of the sample being carried over into the extract. This adulteration did not appear to affect the results appreciably and did lead to a comparison of glycol results from extracted and non-extracted material. The results from non-extracted material were

Determination of Polymethylpentanol

[illegible] Polymethylpentanol (C_{11}, Mol. Wt. 158.28), [illegible] a [illegible] of 3,3-dimethyl glycol ranged from 0.0 to 0.3%, in the fermentation liquors. But the most part the [illegible] constituted little more than a coincidence [illegible] many liquors such coincidences were negligible.

A qualitative test for the carbinol was [illegible] on each liquor according to the method of Rosner and Tollens (1). While eliminated the need for making a definite determination on every liquor since many contained more traces of [illegible]. [illegible] were necessary only on a small proportion of them.

Determination of Methanol from the Sample

For a considerable time after [illegible] the usual [illegible] was [illegible] quoted on all liquors. Though no serious difficulty was encountered in this [illegible], it was time consuming, the occasional formic reaction in part of the sample [illegible] carried over into the [illegible]. This can [illegible] appear to affect the results materially but it [illegible] a [illegible] comparison on [illegible] methanol. The results from [illegible] were

higher than from the extracted and though a correlation existed it was not too good, owing to the variable amounts of sugar and carbinol present. When the appropriate corrections for sugar and acetylmethylcarbinol were applied to the non-extracted material, and not to the extracted, the results agreed fairly well.

At about this time Seagram's method of extraction (4) by means of distilling with kerosene was tried. The results from this method without corrections agreed quite closely with those obtained from the non-extracted material to which corrections had been applied. Because of the unsuitability of our equipment for carrying out routine kerosene extractions, it was decided to estimate glycol directly from the non-extracted sample.

Estimation of 2,3-Butylene Glycol

The periodate oxidation method as described in the original instructions (3) was used for the estimation of butylene glycol. It consisted essentially of oxidizing the glycol with periodic acid to aldehydes, and binding these in a solution of sodium bisulphite. Instead of using 50 cc. of 0.5% periodic acid as suggested, 25 cc. were found to be sufficient for our purposes. After the aldehydes were completely distilled and bound with the bisulphite, the excess bisulphite was titrated with iodine. A portion of carbonate

was then added to break up the aldehyde-bisulphite complex and continued titration with iodine indicated the amount of glycol originally present.

The final titration value was corrected for sugar and acetylmethylcarbinol, using the correction values suggested by the Peoria workers (3).

Adaptation of a Kjeldahl Apparatus

In all three estimations it was found convenient to adapt a twelve-unit electrical Kjeldahl apparatus for distilling and heating purposes. The units ordinarily used for digestion were used in the sugar estimations for boiling the mixtures during the reduction of the copper. The heating units used for distilling were raised about eight inches on a stand. Half of these were used for acetylmethylcarbinol distillations, the other half for butylene glycol. The Hopkin's bulbs were merely fitted with suitable rubber stoppers and, in the case of the glycol estimations, the rubber stoppers were fitted with separatory funnels in order to introduce the periodic acid into the reaction flasks. Otherwise the distillations in both estimations were carried out normally.

Discussion

In most of our work stress has been laid on the production of 2,3-butylene glycol and all experiments and analyses have been conducted with this as the primary viewpoint.

Relatively few ethyl alcohol determinations have been made because it was believed that the alcohol could not be separated satisfactorily from the fermentation liquors. Since this difficulty has been for the most part obviated at the Ottawa laboratory, it is probable that estimations of ethyl alcohol will become a regular part of our analytical program. The oxidative method for determining ethyl alcohol as described by the Ottawa laboratory (4) has also proven satisfactory in this laboratory.

PART II

STUDIES ON AEROBACILLUS

At the outset of the problem it was decided to make a comprehensive investigation of the ability of various organisms to convert carbohydrate material, mainly wheat and wheat products, into 2,3-butylene glycol. Consequently a large number of organisms were isolated and tested for this ability. The more successful were later identified and found to belong to the genus Aerobacillus.

Sources of Organisms

It was first thought that musty cereals or similar carbohydrate material would provide the most likely sources of suitable organisms. Later, however, it was shown that this factor was not important in choosing source material and that promising organisms might be obtained from almost any organic source. Organisms were isolated from 131 distinct sources including all types of manures, feed box scrapings, wheat, alimentary tracts of birds, rotting vegetables, raw and pasteurized milk, spoiled canned goods, various types of decaying vegetative material, numerous soil

types, and a storm-sewer outlet. The storm-sewer outlet is mentioned since the best organism obtained to date (viz., U.A. 233 b) was isolated from this source.

Methods of Isolating and Handling Organisms

The source material was diluted to 1:100,000 to 1:10,000,000 depending on its nature and then plated on starch plates using a nutrient medium made up as follows:

Starch - 1.0%
Beef Extract - 0.3%
Peptone - 0.5%
Agar - 1.5%
Distilled water

The pH of the medium was not adjusted and ranged from 5.1 to 5.5. Selections of all promising colonies were made. In order to eliminate possibilities of contamination iodine was not used on the plate as an indicator until selection was completed. Organisms which had already been selected, but which later showed a negative hydrolysis test with iodine, were discarded.

At first, the method of isolation used included transferring all selections to slants of Potato Dextrose Agar. This medium was a slight modification of Fred and Waksman's Medium 19 (2, p.14), and was made up as follows:

Agar - 15 g.
Potato - 200 g.
Dextrose - 20 g.
Tap water - 1000 cc.

Potatoes were peeled and sliced, placed in the water and autoclaved for one hour at 15 pounds steam pressure. After straining the mixture, nutrients and agar were added and the volume made up to 1000 cc.

From the slants, the organisms were again plated out on starch plates to test for purity. Those continuing to show starch hydrolysis and remaining pure were again transferred to slants and carried in this manner.

For inoculation purposes, 5 cc. of sterile water were added to the slant and, after scraping the organism from the slant, were transferred to the appropriate starch or wheat mash.

The above-mentioned method of isolation was somewhat time consuming and not completely satisfactory, since some organisms which showed no starch hydrolysis proved to be quite active in the mashes. Consequently a second method of isolation was evolved.

The second method included the same dilutions plated on the Potato Dextrose Agar described above. All colonies were picked and transferred to test tubes containing 6% whole wheat mash, previously autoclaved for 20 minutes.

Active tubes were used as a source of inoculum, in

which case 5 cc. of the material were used to inoculate a 100 cc. mash. The active organisms were kept on slants as before.

This system proved satisfactory for a while but it soon became apparent that over a period of time the organism did not maintain its activity on the Potato Dextrose Agar. Following this a different carrying medium was used containing:

Agar	- 17.0 g.
Peptone	- 3.0 g.
Distilled water to make 1000 cc.	

A 0.1-gram sample of ground wheat was placed in each test tube prior to the addition of the agar during the preparation of the slants. This medium has proven reasonably satisfactory provided the cultures are transferred every week.

Types of Mashes

Starch mashes have been the main consideration, partly because of the difficulty encountered in separating the final liquid from the nitrogenous material in wheat mashes, and partly because of the possible market for gluten if it could be satisfactorily separated from the starch before fermentation. A number of wheat mashes have been fermented with consistent results. The influence of nutritional variation is much less marked than in the case of starch mashes.

Details of the Starch and Wheat Mashes

Starch used in the preliminary work was obtained from flour by hand separating the gluten in flowing distilled water. After the washings had settled, the supernatant water was siphoned off and the starch dried at room temperature on sheets of glass. It was then finely ground for use in the fermentations. Later, wheat starch separated mechanically in the Ottawa laboratory became a basis for the mashes.

In preparing the fermentation mash, two litres of 6% starch mixture were heated on an open steam bath at 100° C. 100 cc. portions were then poured into 200 ml. Erlenmeyer flasks and autoclaved for 30 minutes. Following this the inoculum and necessary salts were added.

In the case of the wheat mashes, 10 g. of ground wheat were weighed directly into each 200 cc. flask. Water and salts were added to give a final volume of 125 cc. or an 8% wheat mixture. The material was autoclaved as before.

Effect of Salts

Salts added to each fermentation flask consisted of 1 ml. of 6% $MgSO_4.7H_2O$ and 1 ml. of 6% KH_2PO_4, previously sterilized. It was found that salts sterilized separately

Details of Bran Steam and Heat Studies.

Steam used in the preliminary runs was obtained from flour by wind separating the lumps in flowing distilled water. After the moisture had certain, the separated wheat was dipped at, and the steam dried at that temperature on screens of glass. In one case finely ground for use in the fermentations. Later, wheat starch separated commercially in the Ottawa Laboratory became a basis for the studies.

In roasting the fermentation meal, two lots of 6% moisture were heated on an open steam bath at 100° C. 100 cc. portions were then packed into 500 ml. Erlenmeyer flasks and autoclaved for 2 minutes. Following the inoculum and necessary salts were added.

In the case of the wheat meals, 10 g. of ground wheat were weighed directly into each 500 cc. flask. Water and salts were added to give a final volume of 100 cc. at 8% wheat starch. The method was otherwise as before.

Tests of Yield.

Yields under 5 mgs. per 100 ml. were not [illegible]

and added three to ten hours after inoculation gave much better results than when sterilized together with the mash. This was true particularly of starch mashes.

Effect of Temperature

Experiments in which temperatures of 30° C. and 37° C. were used showed marked differences. The butylene glycol yields at 30° C. ranged from 0.90% to 1.20%, while at 37° C. the values rarely exceeded 0.30%. These results were obtained using various isolates on both wheat and starch mashes.

Effect of Aeration

Aeration was used only in some of the preliminary trials and was found to be non-essential for the Aerobacillus group of organisms.

Effect of Wheat Germ

Probably the most significant effect encountered in the nutritional studies was that of wheat germ. When the addition of wheat germ to the mashes was first tried, no improvement in yields was shown and at times a deleterious

Effect of Temperature

Experiments in which temperatures of 30° C. and 37° C. were used are recorded above. The results differ yields at 30° C. ranges from 0.90% to 1.70%, while at 37° C. the values rarely exceeded 0.30%. These results were obtained using various isolates on both wheat and durum wheats.

Effect of aeration

Aeration was used only in some of the preliminary ...la and was found to be non-essential for the ...

effect was apparent. This was true when the germ was added before sterilization of the mash. It was found, however, that if the organism was allowed to become established, and then the germ added without sterilization, higher yields of butylene glycol could be obtained from the starch mashes.

Sterilizing the germ separately and adding it at the same time as the salts (i.e. 3 to 10 hours after inoculation) was tried with the following results:

	Butylene glycol yield, %
(1) Non-sterile wheat germ added to mash after inoculation	1.26
(2) Wheat germ steam-sterilized for 20 minutes at 15 lb. - germ added after inoculation	1.18
(3) Wheat germ oven-sterilized for 2 hours at 100° C., added after inoculation	1.18
(4) Wheat germ sterilized with mash	poor growth - not analysed

A separate test gave an average glycol yield of 0.70% when the non-sterile wheat germ was added following inoculation and 0.27% when the germ was sterilized with the mash.

A third test included in some fermentations the addition of minor quantities of wheat fractions along with the wheat germ. The results of other tests using additions of wheat fractions will be dealt with later in the report. In this test the wheat fractions were not sterilized and were added to the mash following inoculation. The wheat

germ however was given various treatments, viz.:

(1) non-sterile added to the mash after inoculation;

(2) steam-sterilized added as above;

(3) oven-sterilized added as above;

(4) sterilized with the mash.

The glycol yields are given in Table I.

TABLE I

	Treatments			
Added to the mash	1	2	3	4
0.1 g. Germ No.1*) 0.3 g. Bran (0.3 g. Flour)	0.98	1.00	poor growth	poor growth
0.5 g. Germ No.2*	1.40	1.01	1.11	poor growth
0.5 g. Germ No.2) 0.1 g. Flour (	1.17	0.75	1.20	0.47

* Germ No. 1 was a commercial preparation of practically pure germ, not processed in any way.

* Germ No. 2 was a fraction obtained in the laboratory from an experimental mill and was approximately 15% pure

The above results show that sterilization in itself does not seriously affect the germ since fairly good results are obtained from the sterile germ when it is added separately. However, these results substantiate previous ones in that

sterilization of the germ with the mash greatly reduced the glycol yields.

In general, the results obtained from non-sterile germ added separately have been higher than for either steam-sterilized or oven-sterilized material. This is not indicated very clearly in the above table, probably owing to the relatively large quantities of non-sterile wheat fractions added which are likely to cause contamination or other harmful effects, but it was confirmed in later tests. The addition of non-sterile wheat germ after inoculation has caused no difficulty from the standpoint of contamination, provided the organism is well established before the addition.

The effect of different concentrations of wheat germ in combination with different quantities of urea was tried also. This time a third type of wheat germ (Germ No.3) was used. It consisted of a commercially prepared product from which most of the fat had been removed. The results are given in Table II.

TABLE II

% glycol obtained using varying concentrations of Germ No.3 and urea

Urea - ml. of 2% solution per 100 cc. mash	Germ in gm. per 100 cc. of mash			
	0.0	0.2	0.5	1.0
0.0	0.0	1.10	1.27	1.11
0.05	0.66	0.92	1.17	1.18
0.10	0.94	0.98	1.06	1.23
0.50	0.65	1.08	1.28	1.38

sterilisation of the corn with the weak efficiently reduced the final yield.

In general, the results obtained from non-sterile corn added separately have been higher than for either sterilised or oven-sterilised material. This is not indicated very clearly in the above table, probably owing to the relatively large quantities of non-sterile wheat Tecticin added which are likely to cause contamination or other neutral effects, but it was confirmed in later tests. The addition of non-sterile wheat germ after inoculation has caused no difficulty from the standpoint of contamination, provided the organism is well established before the addition.

The effect of different concentrations of wheat germ in combination with different quantities of urea was tried also. This time a third type of wheat germ (Germ No.3) was used. It consisted of a commercially pre-urea reduct from which most of the fat had been removed. The results are given in Table II.

TABLE II

Effect of urea on yield of germ No.3 and urea

Urea - Gm. of N. solution per 100 cc. broth	Germ in Gm. per 100 cc. of broth			
	0.0	0.3	0.6	1.2
0.0	0.0	1.10	1.44	1.41
0.05	0.86	0.39	1.17	1.12
0.10	0.88	0.42	1.08	1.29
0.20	0.85	1.08	1.18	1.39

At all times when urea was present, increases in wheat germ up to 1.0% gave increases in glycol. No growth was produced when both urea and germ were omitted. Higher concentrations of urea appeared to favor higher concentrations of wheat germ.

Further tests including higher concentrations of wheat germ showed that 1% gave equally as high yields as any other concentration.

An experiment was then set up to determine the effect of varying concentrations of the different types of wheat germ along with varying amounts of urea. The results in Table III again denote the increases in glycol yield with increasing amounts of wheat germ. The effect of different types of wheat germ in this case did not appear significant.

TABLE III

Effect of different types of wheat germ on varying concentrations, along with varying amounts of urea

Urea - ml. of 2% solution per 100 cc. mash		Germ in gm. per 100 cc. of mash			
		0.1	0.2	0.5	0.8
Germ No. 1:	none	0.94	1.01	1.46	1.34
	0.2	0.99	--	1.22	1.42
	0.5	--	1.23	1.41	1.50
Germ No. 2:	none	0.59	0.80	1.09	sample lost
	0.2	0.73	0.80	1.07	1.38
	0.5	0.85	0.79	1.23	1.33
Germ No. 3:	none	--	1.15	1.13	1.33
	0.2	--	1.03	1.27	1.43
	0.5	1.05	1.19	1.16	1.44

Germ No. 2 gave slightly lower yields as might be expected from its impure state. There was no notable difference between No. 1 and No. 3, however, which would seem to indicate that the extraction procedure used in processing No. 3 removed none of the material responsible for increasing the yield of glycol. The blanks in the table indicate poor growth and no samples of these were taken for analysis. One sample was lost during analysis.

To confirm these results further, a sample of wheat was run through a wheat scourer several times in order to remove much of the germ. Kernels whose germs were removed were separated and used as the basis for certain mashes in comparison with others from natural wheat. The average glycol yield from natural wheat was 0.63% compared to 0.30% for germless wheat.

Effect of Carbonates

The need for small quantities of $CaCO_3$ in the fermentations prompted a study of the nature of this effect. The effect of adding different quantities of Na_2CO_3 was also studied in relation to varying amounts of $CaCO_3$.

The $CaCO_3$ added to each flask was weighed, placed in 5 ml. of water and autoclaved. After sterilization, the appropriate quantity of Na_2CO_3 was added. The mixture was then transferred by means of a sterile pipette to the fer-

mentation mixture some time after inoculation. It was found that if the carbonates were added sooner than 12 hours after inoculation there was a deleterious effect. However, from 12 to 24 hours gave no apparent difference.

TABLE IV

Effect of varying amounts of Na_2CO_3 and $CaCO_3$

	$CaCO_3$ - g. per 125 cc. of mash	Na_2CO_3 - g. per 125 cc. mash			
		0.0	0.1	0.2	0.5
Test 1:	0.0	0.52	1.03	1.12	1.10
	0.1	1.09	1.13	1.08	1.24
	0.3	1.16	1.20	1.22	1.17
	0.5	1.21	0.85	1.03	--
Test 2:	0.0	0.46	1.16	1.14	1.12
	0.1	0.96	1.28	0.95	1.05
	0.3	1.14	1.26	1.01	0.98
	0.5	0.96	1.19	1.11	0.73
Test 3:	0.0	0.40	0.92	0.80	0.58
	0.1	1.03	1.10	0.76	0.72
	0.3	0.96	1.18	0.94	0.86
	0.5	1.04	0.91	0.80	0.68

Table IV combines the results of three tests, the first two of which are duplicate tests using organism 233; the third, a similar test using a selection of the same organism (viz. 233b). Most of the figures in this table represent the average of duplicate fermentations.

The highest results were obtained when 0.1 grams of Na_2CO_3 were used, and at this concentration the effect

of variations in $CaCO_3$ was not great. In general, the optimum amount of $CaCO_3$ appeared to be 0.3 grams. Higher concentrations of both Na_2CO_3 and $CaCO_3$ had definite deleterious effects.

In more concentrated starch mashes it was necessary to increase the concentrations of Na_2CO_3 and $CaCO_3$ also. This suggests that at least part of the effect of carbonates is a buffering effect. This assumption was substantiated by trials using KH_2PO_4. 0.5 cc. of 6% KH_2PO_4 added at the time of inoculation gave glycol yields of 1.45% compared with 0.99% when the KH_2PO_4 was added at the same time as the $CaCO_3$. The KH_2PO_4 supplies buffering right from the start and increases here shown warrant further study of this effect.

The addition of $Ca(H_2PO_4)_2$ in an endeavor to supply both a buffering agent and the calcium ion was not successful and in all cases where this substance was added low glycol yields resulted.

Effect of Adding Wheat Fractions

Although additions of wheat germ were found to increase the glycol yields appreciably, the amounts necessary to carry out such additions on a commercial scale would be extremely difficult to obtain. Consequently the addition of other wheat fractions was studied to determine whether or not they might replace the beneficial factor present in the

germ. One of these tests was carried out in combination with variations in $CaCO_3$. 0.2 g. of the particular wheat fraction was added to each mash. The results appear in Table V.

TABLE V

The effect of additions of different wheat fractions at two levels of $CaCO_3$

Fractions	0.1 g. Na_2CO_3 0.1 g. $CaCO_3$	0.1 g. Na_2CO_3 0.3 g. $CaCO_3$
Wheat	0.96	1.06
Shorts	0.96	0.99
Feed flour	1.08	1.12
Patent flour	0.99	1.22
Germ No. 2	0.96	1.05
Germ No. 1	1.14	1.32

The beneficial effect of the larger amount of $CaCO_3$ is indicated. Germ No. 2 as obtained from the mill fractions was no more beneficial than the other fractions and was inferior to the flours. The purer Germ No. 1, however, gave definitely higher yields than any of the mill fractions.

Further experiments using varying amounts of bran and flour by themselves and with wheat germ showed that glycol yields could be increased by additions of mill fractions, though to a lesser extent than with wheat germ. Concentrations of bran above 0.5% gave a reduction in glycol yield. The reduction was not so apparent with flour. As

germ. One of these tests was carried out in a complete set of variations in $CaCO_3$. 0.5 g. of the particular germ fraction was added to each mean. The results are given in Table V.

TABLE V

The effect of additions of different germ fractions at two levels of $CaCO_3$.

Fractions	0.1 g. $CaCO_3$	0.5 g. $CaCO_3$
Sheet	0.96	1.00
Shorts	0.96	0....
Feed flour	1.08	1.11
Patent flour	0.90	1....
Germ No. 2	0.98	1....
Germ No. 1	1.14	1.06
		1.06

The beneficial effect of the lower grade of $CaCO_3$ is indicated. Germ No. 2 as obtained from the mill fractions was no more beneficial than the coarse fraction and was inferior to the flours. The purer germ no. 1, however, gave definitely higher yields than any of the mill fractions.

Further experiments using varying amounts of germ and flour by themselves and with wheat germ showed that typical yields could be increased by additions of mill fractions, though to a lesser extent than with wheat germ. Concentrations of bran above 0.5 g. gave a reduction in typical yield. The reduction was not so apparent in flour. In

for wheat germ added in a natural condition, no pronounced inhibiting effect could be detected in the higher concentrations.

Discussion

Most of the technical difficulties encountered in eliminating contamination, preparing a satisfactory inoculum and maintaining vigor of the isolated organisms have been successfully combatted.

It is felt that the maintenance of a proper pH is important and that proper buffering would be advantageous in obtaining consistently high yields of glycol, particularly in the starch mashes.

The effect of nutrition is apparently an important one. A factor present in wheat fractions and concentrated in the wheat germ had a definite beneficial effect. Since the wheat kernel contains little more than 1% germ and since the germ is not easily separated from the remainder of the kernel, the practice of adding wheat germ to fermentations does not become feasible on a commercial scale. Study in relation to the nature of this beneficial factor has been considered but little work has been done in this connection as yet. If starch mashes are to be used, work on this phase is certainly warranted.

In general, it is now possible to duplicate fermentations and experiments with a reasonably low error.

The yeast germ added in a natural condition, no pronounced [illegible] effect could be detected in the flavor consumers.

Conclusion.

Most of the chemical difficulties encountered in eliminating contamination, preparing a marketable [illegible] any marketable value of the isolated germs have been [illegible].

It is felt that the performance of a flavor [illegible] important and that proper buffering would be advantageous in obtaining consistently high yields of flavor, particularly in the [illegible].

The effect of rancidity is apparently an important [illegible]. A factor present in wheat treatment and concentrated in the wheat germ had a definite beneficial effect. Since the wheat kernel contains little more than 1% germ and since the germ is not easily separated from the remainder of the kernel, the practice of making wheat germ [illegible] does not become feasible on a commercial scale. Study in relation to the nature of this beneficial factor has been considered but little work has been done [illegible].

In general, it is now possible to [illegible] [illegible].

PART III

STUDIES ON AEROBACTER AEROGENES

In May, 1942, four pure cultures of organisms were obtained from Ottawa and tested according to the methods described in the original report of the Peoria Conference, April, 1942. These organisms were:

(1) Aerobacter aerogenes (No. 199)

(2) Aerobacter oxytocum (BT-1)

(3) Bacillus cloacae (BT-3)

(4) Escherichia Coli (BT-2)

After a few comparative tests using these organisms on glucose fermentation medium Aerobacter aerogenes was chosen as the superior organism, and with the exception of a few trials using unknown organisms was used in all subsequent work.

Purpose of the Study

Aerobacter aerogenes is a very efficient organism for the purpose of converting glucose to 2,3-butylene glycol. It has little or no ability, however, to convert or hydrolyse starch and is relatively sensitive to the factors which affect its activity.

The purpose of this study was to make a comprehensive survey of the factors affecting the activity of this or

Page III

In May, 1942, four pure cultures of organisms were obtained from Odessa and tested according to the methods described in the original report of the Textile Conference, April, 1941. These consisted of:

(1) Chromobacter violaceum (No. 149)

(2) Aerobacter cyclogenum (Ac-1)

(3) Bacillus alcaligenes (BA-3)

(4) Pseudomonas fluorescens (PF-3)

After a few preliminary tests using these organisms as the sporing organism, and with the exception of a few trials using unknown organisms were used in all succeeding work.

Purpose of the Study

Aerobacter cyclogenum is a very efficient organism for the purpose of corrosion pickoff to 1,4-butylene glycol. It has little or no utility, however, to convert to butylene glycol and is relatively sensitive to the textile weight effect its activity.

The purpose of this study was to make a preliminary survey of the factors affecting the activity of this or

other organisms with a view to obtaining a complete and rapid conversion of wheat or its products to 2,3-butylene glycol. The use of such carbohydrate material would necessitate, of course, hydrolysis of starch to glucose and much of our work to date has been concerned with this phase.

Methods of Handling and Carrying the Organism

Slants of Aerobacter aerogenes were stored in a refrigerator except when they were needed, and then they were stored at room temperature. The medium used for the slants was the agar medium suggested by the Northern Regional Research Laboratory (4), made up as follows:

Difco tryptone	- 5 g.
Glucose	- 1 g.
Difco yeast extract	- 5 g.
Agar	- 15 g.

Distilled water to make 1000 cc.

Several months after the start of the project there was reason to believe that the organism had degenerated probably owing to frequent transferring or improper storage conditions.

An experiment conducted to determine degenerative effect gave the following yields of butylene glycol*. The

* The yields of glycol reported here and throughout the remainder of the report represent the percentage glycol obtained compared to the theoretical amount obtainable from sugar consumed.

figures given represent the average of four fermentations:

(1) An original waxed slant stored in the refrigerator for 8 months without transfer being made ..... Average - 90%

(2) An original slant, unwaxed, kept at room temperature, transferred once and used ..... Average - 89%

(3) Slant transferred approximately every second week for eight months and stored in a refrigerator ..... Average - 95%

(4) Original slant obtained from Ottawa, stored for eight months, transferred twice in one week and used ..... Average - 99%

The above results would seem to indicate that appreciable degeneration had not taken place. On the average a higher conversion was obtained from slants freshly transferred.

Inoculum Studies

The inoculum medium used was also that suggested by the Northern Regional Research Laboratory (4), containing:

Commercial refined glucose	- 100 g.
Concentrated corn steeping liquor	- 5 g.
$CaCO_3$	- 5 g.
Distilled water to make	1000 cc.

Replacing the commercial refined glucose with glucose obtained as the result of starch hydrolysis gave a medium inferior to that suggested, so that the medium described above was used throughout.

To develop the inoculum, 10 cc. of the prescribed medium were inoculated from a slant and incubated without aeration for 24 hours at 30° C. It was then ready for inoculation into the fermentation medium.

The Peoria report (4) suggested that the fermentation medium should be inoculated with 5 to 10% of its volume of 24-hour-old inoculum. An experiment using various amounts of inoculum gave the following results:

	Average of four fermentations
(1) 6% inoculum added	78.5%
(2) 10% inoculum added	85.5%
(3) 20% inoculum added	95.5%

The above figures represent fermentations on a 6.5% dextrose solution, the quantity of fermentation mixture being 50 cc. including the inoculum. In this case increasing the amount of inoculum gave very definite increases in the glycol conversion.

Varying the age of the inoculum from 12 to 36 hours gave no apparent difference in the results.

	Average of four determinations
(1) 5% inoculum added	72.5%
(2) 10% inoculum added	74.0%
(3) 20% inoculum added	76.5%

Fermentation Media

Most of the experiments were carried out on a hydrolysed starch medium but were also run on a glucose fermentation medium to give a basis of comparison.

The glucose fermentation medium was made up as follows:

Commercial refined glucose	-	100-200 g.
$MgSO_4.7H_2O$	-	0.25 g.
KH_2PO_4	-	1.80 g.
Urea	-	2.0 g.
$CaCO_3$	-	5.0 g.
Distilled water to make		1000 cc.

The glucose, $MgSO_4.7H_2O$ and KH_2PO_4 were first dissolved and sterilized. The urea was sterilized separately as a 2% solution, and added at the time of inoculation. The $CaCO_3$ was sterilized dry and added at the time of inoculation.

Varying the amounts of these constituents had little or no effect provided these constituents were present in sufficient quantities to meet the minimal requirements of the organism. At least 0.1 g. of $CaCO_3$ was required. Na_2CO_3 did not increase the yields as in the case of Aerobacillus. It was found that urea as a source of nitrogen could be replaced by wheat germ or bran but these presented practical difficulties.

Considerable work was conducted in an endeavor to

Fermentation media.

Most of the experiments were carried out on a modified yeast medium but were also run on a glucose fermentation medium to give a basis of comparison.

The glucose fermentation medium was made up as follows:

Commercial refined glucose -	100-200 g.
$MgSO_4.7H_2O$ -	0.25 g.
$(NH_4)_2SO_4$ -	1.0 g.
Urea -	2.0 g.
KH_2PO_4 -	1.0 g.
Distilled water to make	1000 cc.

The glucose, $MgSO_4.7H_2O$ and $(NH_4)_2SO_4$ were first dissolved and sterilized. The urea was sterilized separately as a 5% solution, and added at the time of inoculation. The KH_2PO_4 was crystalline and added as the dry salt.

Table [illegible] gives the results obtained with various quantities of these fermentation media. At least 0.1 g. of KH_2PO_4 was required per 100 cc. of medium. Above this quantity the yields did not increase the yields as in the case of [illegible].

It was found that urea as a source of nitrogen could be replaced by wheat germ or bran but these products [illegible] nitrogen.

Considerable work was conducted in an endeavour to

obtain a suitable fermentation medium based on the products of starch hydrolysis. For this purpose, wheat starch similar to that used in the studies on Aerobacillus was hydrolysed by two methods: (1) by low pressures (2) by high pressures.

(1) Low-pressure Method

This procedure embodied essentially the hydrolysis of starch in an autoclave, using HCl. It was found difficult in laboratory procedure to convert relatively concentrated starch mixtures into sugar without the occurrence of caramelization. The procedure finally recommended was to autoclave a 15% starch mixture in 0.2 NHCl for one hour at 15 pounds of steam pressure (121° C.). The starch was first made up with water to 15% and then heated to a boil in order to convert the starch to a more soluble form. Sufficient HCl was then added to give the proper acidity for hydrolysis. Following the hydrolysis the acid solution was neutralized with CaO, decanted aseptically into a large stock bottle, and used as required. Some of the precipitate is eliminated in the decantation. CaO, rather than $CaCO_3$, was used for neutralization to prevent foaming. In addition, it was impossible to obtain a sufficiently high pH using $CaCO_3$ alone.

By this method reasonably large quantities of uniform material were obtained for experimental purposes.

(2) High-pressure Method

Here, a 10% starch solution was prepared by pouring a cold paste of starch and water into a boiling solution of 0.2 N HCl, with stirring. One-litre lots of this solution were placed in a pressure heater, the temperature brought to 170°-180° in three or four minutes and held for two or three minutes. By dropping the pressure rapidly, minimum "browning" took place and better conversion was obtained than with the low-pressure method. However, the pressure heater was adapted for small batches only and was not used extensively on this account. Neutralization of the hydrolysate was carried out the same way in both methods and when used as a fermentation medium, salts and urea were added in the same manner as in the glucose fermentation medium.

A few tests have been conducted using as a base ground wheat hydrolysed with acid. No significant results on this phase have been obtained to date.

Effect of Variation in Conditions

(1) Temperature

Fermentation carried out at 27°, 30°, 33°, and 35° C. showed that while appreciable fermentation took place at all of these temperatures, the percentage conversion to

(3) Digestion Bombs

Effect of Variation in Conditions

(1) Temperature.

glycol was highest at 30° C. Temperatures below 30° C. were less detrimental than those above 30° C. This is in agreement with results obtained at Peoria.

(2) Aeration

It was found that fermentations using Aerobacter aerogenes required aeration at all times. The workers at both Peoria and Ottawa have suggested discontinuing the aeration after the sugar concentration has dropped below a certain level. This has not been found advisable in our laboratory and consistently better results have been obtained by continuing moderate to heavy aeration until fermentation has been completed. Despite persistent aeration our yields of acetylmethylcarbinol have always been negligible for Aerobacter aerogenes fermentations.

Certain aeration studies conducted gave the following results:

	Average of four fermentations
(1) Moderate aeration for four days. Allowed to stand one day without aeration	92%
(2) Moderate aeration for four days then analysed	78%
(3) Moderate aeration for five days	95%
(4) Heavy aeration for five days	98%

The above figures indicate that the fermentation was apparently not complete at the end of four days. Heavy aeration up to 500 cc. per litre per minute was certainly not detrimental but gave the best results. Poor results were obtained when less than 50 cc. of air per litre per minute was supplied. Above this level, the variations in results were not great but they did appear to favor the heavier aeration.

(3) Methylene Blue

Since aeration is somewhat difficult to control and presents some physical difficulty when applied on a commercial scale, the field of substitutes was explored. It was thought that if the function of the air was an oxidative one then it could be replaced by some hydrogen acceptor such as methylene blue. The addition of varying quantities of methylene blue in place of aeration gave results ranging from 5% to 50% theoretical glycol. Results higher than this could not be obtained and would seem to indicate that aeration performs a function in addition to the oxidative one. The Ottawa laboratory has obtained fairly good results by replacing the air with nitrogen which would suggest that agitation of the fermenting mixture is one of the functions of aeration.

Studies on Unknown Organisms

In addition to the work on Aerobacter aerogenes, a

search was made in an endeavor to obtain an organism which would efficiently convert glucose to 2,3-butylene glycol without aeration. Many of the isolates referred to in Part II were inoculated into glucose medium. Out of 155 organisms tested, only seven gave promising results. These yielded from 58% to 74% theoretical glycol. This phase, therefore, would seem to warrant further study.

Discussion

From the studies to date on Aerobacter aerogenes, it is clear that the organism under proper conditions can be made to produce almost solely 2,3-butylene glycol from glucose. The organism has certain disadvantages including the necessity for aeration, and saccharification of starch and also it will maintain active fermentation only under quite specific conditions.

In our laboratory most of the influencing factors have been controlled sufficiently to give satisfactory conversions, but as yet we have been unable to attain the rapid rate of fermentation reported by other workers. While the regular duration of fermentation in other laboratories is 40 to 60 hours, our fermentations are not generally completed under 100 hours.

The hydrolysis of starch which has been one of our problems has been satisfactorily worked out in the alcohol

industry and should not present extreme difficulty in commercial fermentations using Aerobacter aerogenes.

If a high glycol:alcohol ratio were desirable in the fermentation products then an organism of the Aerobacter aerogenes group would be desirable, and an organism which would perform essentially the same conversion without aeration would be particularly advantageous.

On the other hand, assuming that fermentations with a lower glycol:alcohol ratio may be utilized in view of the satisfactory recovery of alcohol from the mixture, then an organism of the Aerobacillus group which would perform the saccharification and give a reasonably high yield of total products, would undoubtedly be the leading organism.

SUMMARY

From July, 1942, until February, 1943, approximately 2,000 fermentation liquors were obtained and analysed for 2,3-butylene glycol. Of these 1,000 were fermentations of ground wheat starch, using Aerobacillus. An additional 500 fermentations using Aerobacillus were conducted on ground whole wheat. The remainder were conducted on hydrolysed starch and on pure glucose using Aerobacter aerogenes. In general, fermentations of wheat gave higher percentage conversion of starch to glycol than did the pure starch fermentations.

The periodate oxidation method for determining 2,3-butylene glycol was used throughout. It was performed on the supernatant liquid of the liquors and the results obtained were corrected for reducing sugar and acetylmethylcarbinol present.

Aerobacillus fermentations yielded up to 50% of the theoretical butylene glycol from both wheat and starch mashes.

Additions of Na_2CO_3 along with $CaCO_3$ increased the yield of glycol somewhat. Additions of wheat germ to the fermenting mash gave significantly higher yields of glycol but the effect of these additions on the glycol:alcohol ratio was not determined.

Glucose fermentations using Aerobacter aerogenes gave glycol yields approaching theoretical percentages. The conditions under which this organism maintains maximum fermenting ability were found to be quite sensitive. In general the Aerobacter fermentations proceeded slowly, probably owing to an inadequate method of aeration.

The products of starch hydrolysis were found to supply a fermentation medium comparable to that of refined glucose. A satisfactory laboratory method of starch hydrolysis was worked out and is presented here in detail.

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